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14. ABSTRACT

Loss of G2/M checkpoint plays an important role in tumorigenesis, however, few genes involved in this checkpoint control have been shown to be deregulated in human breast tumors. SAFB1 is a multifunctional protein which maps to a locus of high LOH, and mutations have been identified from both breast cancer cell lines and tumors. Our preliminary data show that inactivation of SAFB1 in MEFs result in loss of G2/M checkpoint control, and that loss of SAFB1 expression is associated with Taxotere resistance in human breast tumors. We therefore hypothesize that SAFB1 is critical for G2/M checkpoint control, and that its inactivation results in resistance to breast cancer therapies that utilize a block in G2/M and subsequent apoptosis. We will identify the mechanism by which SAFB1 controls the G2/M checkpoint, and will subsequently analyze whether Taxotere-resistant tumors show altered expression of genes involved in these pathway(s).

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

SAFB, Taxotere resistance, checkpoint, apoptosis, MEFs

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Introduction:

SAFB1 is a multifunctional protein which maps to a locus of high LOH, and mutations have been identified from both breast cancer cell lines and tumors. Our preliminary data for the grant showed that inactivation of SAFB1 in MEFs results in loss of G2/M checkpoint control, and that loss of SAFB1 expression is associated with Taxotere resistance in human breast tumors. We therefore hypothesize that SAFB1 is critical for G2/M checkpoint control, and that its inactivation results in resistance to breast cancer therapies that utilize a block in G2/M and subsequent apoptosis. We proposed to identify the mechanism(s) by which SAFB1 controls the cell cycle checkpoint(s), and to analyze whether drug-resistant tumors show altered expression of genes involved in these pathway(s).

Body:

The progress made in Aims 1 through 3 is outlined below:

Aim 1) Is the G2/M checkpoint lost in mammary epithelial cells from the SAFB1$^{-/-}$ knockout mice?

Progress:

Our data showed that loss of SAFB1 was associated with a loss of G2/M checkpoint control in MEFs. To make this work more relevant to human disease, we had proposed to study mammary epithelial cells from the SAFB1-/- mouse model. However, we have discovered that the knockout of SAFB1 in the mouse results in dramatic systemic effects in the mice (such as altered IGF and hormone levels) which would make it difficult to interpret any results from the SAFB1-/- MECs. Therefore, we needed to set up a different model system. We have initiated a collaboration with Dr. Paul Yaswen (LBL, Berkely, CA), who is an expert in the use of "normal" human epithelial cells, and we will use our SAFB1 siRNA to decrease endogenous SAFB1 expression in human mammary epithelial cells (HMECs). This work is currently ongoing.

Interestingly, our studies with the MEFs revealed that there is not only a defect with the G2/M checkpoint control, but SAFB1 loss also resulted in extended length of S-phase (Fig 1). These data indicate that SAFB1 might also play a role in S-phase progression.

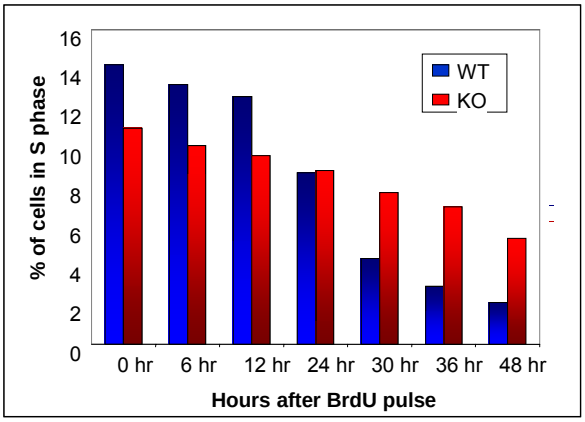

Fig 1: Unsynchronized SAFB1 wildtype and knockout MEFs were pulse-labeled with BrdU for 1 hr, and BrdU incorporation was measured at indicated hours (x-axis).

In collaboration with Dr. Rao Pulivarthi, we have analyzed whether loss of SAFB1 results in polyploidy and chromosome missegregation. This work has been finished using MEFs, and we did not detect any

reproducible and consistent genetic defects (Fig. 2). Once we have confirmed cell cycle checkpoint defects in HMECs with downregulated SAFB1 expression, we will repeat the studies in this human epithelial cell line model.

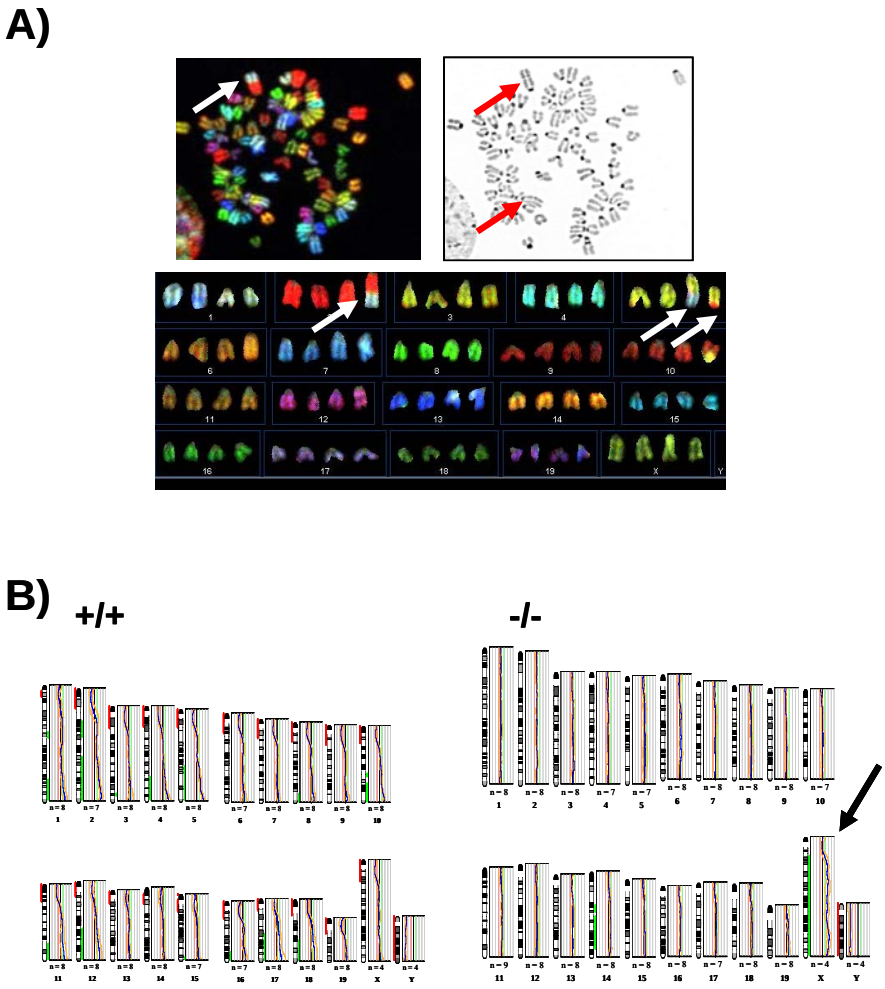

Figure 2: Analysis of genomic instability in MEFs.

A: Spectral karyotyping (SKY) performed on immortalized SAFB1-/- MEFs (passage 27) revealed several translocations (as indicated by arrows), however, this result was not reproducible in the second experiment. Top left panel: spectral image of SAFB1-/- MEFs; top right panel: inverse DAPI images; bottom panel: computer-classified images. In the computer-classified image, the arrows indicate the following translocation (from left to right): (2;5), t(5;1), and t(5;2).

B: Comparative genomic hybridization (CGH) performed on immortalized wild-type (n = 1) and SAFB1-/- (n = 3) MEFs did not reveal significant abnormalities in SAFB1-/- MEFs, except for the amplification of the X chromosome (as indicated by the arrow). Subsequent FISH analysis, using a number of different SAFB1+/+ and –

/- clones, did not confirm X-chromosome amplification Amplifications are represented by green bars to the right of the chromosome ideogram, while deletions are indicated as red bars to the left of the ideogram.

PWe have also begun to test whether loss of SAFB1 has an affect on sensitivity to other frequently used cytotoxic drugs. Specifically, we have tested the following drugs: doxorubicin, etoposide, 5-FU, vinblastine, and mitoxantrone. Interestingly, the SAFB1-/- MEFs are more susceptible to 5-FU and mitoxantrone, an effect which we currently do not understand. These experiments are being repeated, and we hope that mechanistic studies in Aim 2 will help to explain these unexpected observation.

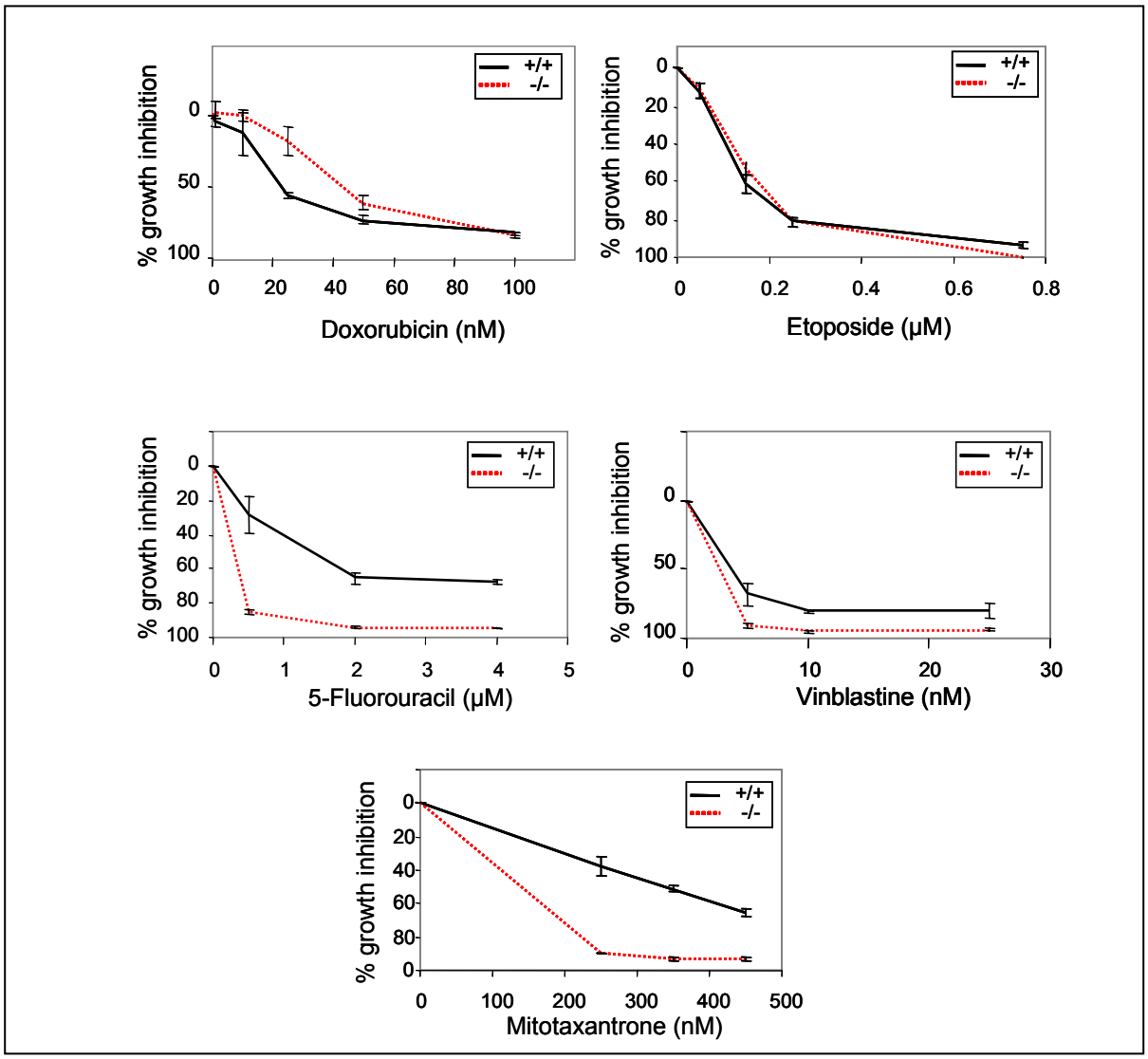

Figure 3. Analysis of SAFB1-/- MEFs' sensitivity to various chemotherapeutic drugs. MEFs stably transformed with Ras/Myc were incubated in the presence of various drugs at indicated concentrations, and

growth was measured on day 3. A representative clone is shown. Each data point represents average of a triplicate well.

Aim 2) What is the mechanism for the SAFB1-mediated G2/M checkpoint control?

Progress:

As described in last year's progress report, we had identified that the tumor suppressor and cell cycle inhibitor p19ARF was lost in SAFB1-/- MEFs. We have obtained additional data showing that the loss of p19ARF was not due to direct affects of SAFB1 on p19ARF promoter activity, but were indirect. SAFB1 can inhibit a known repressor of p19ARF, the T-box transcription factor TBX2 (1), therefore ultimately resulting in loss of p19ARF in SAFB1-/- cells. This is of special interest for breast cancer, since TBX2 has been described a) to play a major role in mammary gland development, and b) to be amplified in breast cancer (2). These studies have been submitted to Cancer Research for publication.

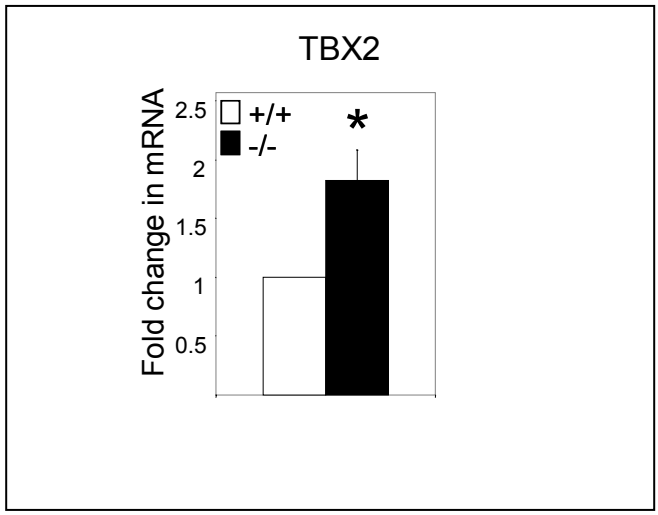

Figure 3. Increased TBX2 levels in SAFB1$^{-/-}$ MEFs. Analysis of TBX2 in primary SAFB1$^{-/-}$ MEFs by QPCR. TBX2 mRNA levels in SAFB1$^{+/+}$ and SAFB1$^{-/-}$ MEFs (n=6) were measured by Q-PCR. The graph represents relative mRNA levels corrected for β-actin. (* p=0.0281; two-tailed paired t-test). Bars represent the mean (n=6) ± SEM

We now know that SAFB1 is not only involved in G2/M cell cycle checkpoint, but that it also plays a role in S-phase (see Aim 1). To understand the obviously complex role of SAFB1 in the cell cycle we decided to perform microarray analysis (using SAFB1+/+ and -/- MEFs) in order to understand the underlying mechanism(s). While the detailed analysis and confirmation of the data is ongoing, we can already say that loss of SAFB1 leads to global deregulation of a number of homeobox genes. We are currently confirming these results, and are planning experiments to determine if, and if so how, homebox genes are related to SAFB1's role in cell cycle.

Other ongoing experiments: Over the last year, we have successfully generated stable SAFB1-/- clones which overexpress various deletion constructs of SAFB1 (ΔRD (repression domain), ΔSAF-Box (scaffold attachment factor Box – DNA binding), ΔRRM (RNA recognition motif)). These clones are currently characterized, and we expect that these studies will help us to understand the mechanism(s) by which SAFB1 participates in cell cycle regulation.

Finally, we have begun to perform confocal microscopy studies in order to perform colocalization studies of SAFB1 with various cell cycle proteins. These studies are ongoing, and we expect them to be finalized in year 3.

3) Are genes which are mechanistically linked to SAFB1-mediated checkpoint control also deregulated in Taxotere-resistant breast cancer?

Progress:

The available patient material from Dr. Chang's neoadjuvant trial is very limited , and we will await the final results from the microarray study before using this valuable resource. These studies will be performed in year 3.

Key Research Accomplishments in Year 2:

1) No reproducible genetic instability in SAFB1-/- MEFs
2) Spontaneous immortalization of SAFB1 KO MEFs associated with loss of $p19^{ARF}$ is likely a result of SAFB1's affect on the T-box factor TBX2, a known repressor of p19ARF
3) Effect of SAFB1 on drug resistance or sensitity is strictly drug-dependent
4) Generation of cell line clones that overexpress SAFB1 deletion constructs to be used to decipher mechanisms suggesting that loss of p19ARF could contribute to decreased apoptosis
5) Defect in S-phase in SAFB1-/- cells (in addition to previously reported defect in G2/M checkpoint)

Reportable Outcomes

Manuscripts:

Klaudia M. Dobrzycka, Kaiyan Kang, Shiming Jiang, Rene Meyer, Rao Pulivarthi, Adrian V. Lee, and Steffi Oesterreich. Disruption of Scaffold Attachment Factor SAFB1 leads to TBX2 upregulation, lack of $p19^{ARF}$ induction, lack of senescence, and cell immortalization. Submitted to Cancer Research.

Klaudia M. Dobrzycka, Kaiyan Kang, Shiming Jiang, Rene Meyer, Adrian V. Lee, and Steffi Oesterreich. Loss of SAFB1 results in G2/M checkpoint defects, associated with rsistance to Taxotere I breast cancer. Manuscript in preparation.

Conclusions:

While we had confirmed in Year 1 that loss of SAFB1 results in resistance to Taxotere (docetaxel), and a loss of a G2/M checkpoint, we have now established that SAFB1 has an additional significant affect on the S-phase of the cell cycle, too. This might explain the

varying effects we have observed using cytotoxic drugs. A series of experiments are ongoing to decipher the underlying mechanism. We have identified some candidate genes, such as homebox genes and TBX-2, but will wait for the completion of these mechanistic studies before we will use the valuable clinical material for experiments proposed in Aim 3.

In summary, we have provided further evidence that SAFB1 is a critical player in cellular immortalization and transformation, and expect to have unraveled the mechanistic basis for these processes in the near future.

References:

1. Jacobs, J. J., Keblusek, P., Robanus-Maandag, E., Kristel, P., Lingbeek, M., Nederlof, P. M., van Welsem, T., van de Vijver, M. J., Koh, E. Y., Daley, G. Q., and van Lohuizen, M. Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat Genet, *26:* 291-299., 2000.
2. Rowley, M., Grothey, E., and Couch, F. J. The role of Tbx2 and Tbx3 in mammary development and tumorigenesis. J Mammary Gland Biol Neoplasia, *9:* 109-118., 2004.